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The "One Source" cohort – evaluating the suitability of the human toenail as a manganese biomonitor

J. M. Guthrie,* J. D. Brockman, J. S. Morris, J. D. Robertson

University of Missouri Research Reactor, Columbia, MO 65211, USA

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Numerous studies have demonstrated that the human toenail is a reliable biomonitor for the intake of Se and other elements. The objective of this study was to evaluate the use of the toenail as a biomonitor for Mn. Toenail specimens from One Source™ multivitamin users and matched controls were selected. Se was measured using established NAA methods, then Mn was measured via a new procedure. The Se results confirmed the accurate classification of the cohort. However, the nail did not show significant, positive response to Mn supplementation. We hypothesize that the persistence of exogenous Mn confounded the results.

Introduction

Manganese is an essential trace element in humans. It has important roles in osteogenesis and is incorporated into enzymes responsible for the reduction of oxidative stress and in amino acid, cholesterol, and carbohydrate metabolism. A recommended daily allowance (RDA) has not been issued for Mn. However, the National Academies' Institute of Medicine has set an adequate intake (AI) of 2.3 mg/d for men and 1.8 mg/d for women.[1] In excess, Mn causes a neurodegenerative disorder known as manganism which results in symptoms very similar to Parkinson's disease.[2,3] The similarities between the two disorders can be partially explained by the fact that the basal ganglia accumulate most of the excess Mn in manganism and dysfunction in the basal ganglia is also the etiology of Parkinson's disease. Manganese deficiency has not been clinically recognized in humans, but in animal studies it has been shown to cause impaired growth, skeletal problems, reduced fertility, abnormal glucose regulation and altered lipid and carbohydrate metabolism.[3–5]

Recently there has been considerable interest in the gene coding of the mitochondrial antioxidant manganese superoxide dismutase (MnSOD) which converts the superoxide radical into water and hydrogen peroxide.[6–9] The selenium-containing enzyme, glutathione peroxidase (GPX) converts the hydrogen peroxide into water. Several research groups are examining the relationship between carcinogenesis and polymorphisms of the MnSOD gene. It has, for example, been shown that replacement of valine (V) with alanine (A) at codon 16 in the human MnSOD gene increases the functionality of MnSOD. Individuals with the AA genotype are better able to import MnSOD into the mitochondria than individuals with the VA or VV genotypes and several studies have suggested an increased risk of cancer with the AA genotype.[8,10,11] The increased cancer risk with the AA genotype is attributed to an increase in hydrogen peroxide production overwhelming the capacity of the GPX enzyme.

At present, little is known about the interactions between Mn and Se status and the interplay of these two trace nutrients in human health. One challenge facing epidemiologists working in this area is a scarcity of Mn biomonitors. The current biomonitors for Mn exposure were recently reviewed by GREGER.[12] Manganese in blood serum is typically found in the concentration range of 0.8 to 2.1 μg Mn/l. At these low concentrations, Mn contamination from stainless steel needles and plastics can contribute to a significant fraction of the measured Mn concentration in the blood sample. Moreover, while Mn is present in urine and bileary secretions, their use as biomonitors are questionable because of complicated absorption and excretion processes.

One possible Mn biomonitor is the toenail.[13,14] Toenails are common, noninvasive biomonitors that are easy to collect and store. The nail, which consists largely of keratinacious proteins, has been shown to accurately monitor dietary intake of the essential trace element Se and the toxic trace elements arsenic and mercury.[15–21] Because of the slow growth cycle of the nail, the trace-element content of the nail can reflect dietary intake integrated over several months. However, while numerous studies have demonstrated increased levels of Mn in both human nail and hair from environmental Mn exposure, there is, to the best of our knowledge, no study in the literature which quantifies the nail response to a controlled dietary Mn stimuli.[13,14,22–25] The objective of this work was to evaluate toenails as a biomonitor for Mn dietary supplements in a treatment-control study.

* E-mail: guthriejm@missouri.edu

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Experimental

Toenail samples were drawn from the Columbia Tribune Study (CTS), which consists of over 3000 toenail specimens collected in 1999 largely from subjects living in the city of Columbia, Boone County, and six adjacent counties in central Missouri.[26] Participants in the CTS voluntarily responded to a local newspaper advertisement, donating nail samples and filling out a questionnaire that included demographic and dietary information. The participants self classified themselves as vitamin supplementers or non-supplementers and answered questions about sex, age, weight, height, race, smoking status, type of vitamin supplement used, and the length of time the vitamin supplement was taken. No follow up was done to confirm the questionnaire answers. Because the advertisement for the CTS followed a series of newspaper articles on dietary Se status and chronic human disorders, the study resonated with a health-conscious segment of the population. Consequently, the percentage of participants who routinely used dietary supplements was greatly over-represented in the study. The One Source multivitamin, manufactured by the Perrigo Company in Allegan, Michigan, was a popular supplement that contained super-nutritional amounts of Se and Mn. The One Source Vitamin, as formulated in 1999, contained 375% of the Daily Value (DV) of Mn and 286% of the RDA of Se.

Test subjects from the CTS who routinely used the One Source Multivitamin (+OS) were matched with individuals who did not take a dietary supplement (–S). Matching criteria were sex, age, body mass index, and smoking status (never, past, current). The cohort for the study (Table 1) consisted of 74 female subjects (37 +OS and 37 –S) and 52 male subjects (26 +OS and 26 –S). All 126 subjects had an archived nail sample that had previously been analyzed for Se. Of these, 62 had supplemental nail sample material archived in the original collection envelope that was used as blinded splits for the analysis. In addition, 20 nail samples that consisted of five replicate samples from four nail collections over 9 months from one individual were also included in the analysis.

The nail samples were sonicated for 10 minutes in 10 ml of 10% (v/v) nitric acid solution, decanted and then sonicated for 10 minutes in 10 ml of 18 MΩ·cm water. The samples were then rinsed with 18 MΩ·cm water and stored in pre-cleaned plastic liquid scintillation vials. The samples were freeze dried using an ATR Heto vacuum freeze drier and then weighed. The masses of the 208 samples ranged from 2 to 80 mg; the median sample mass was 48 mg.

The One Source cohort samples were analyzed for Se by instrumental neutron activation analysis (INAA) in April, 2005. The samples were placed in 0.4-ml high-density polyethylene vials and irradiated in a pneumatic tube system for 7 seconds at a flux of ca. $5.0 \cdot 10^{13}$ $n \cdot cm^{-2} \cdot s^{-1}$. The samples were then allowed to decay for 15 seconds before counting on the face of an HPGe coaxial detector for 30 seconds, real time, using a loss-free pulse pile-up correction. The detector had a measured relative efficiency of 31% and a FWHM resolution of 1.69 keV at 1.33 MeV. All samples had a dead time of less than 5%. The peak areas were determined interactively with Genie ESP spectroscopy software package (Canberra). Comparator standards were prepared by pipetting approximately 0.5 μg of Se from a certified Se Standard (Spex) onto filter paper in a 0.4-ml high density polyethylene vial. Eleven samples of NIST SRM 1577 Bovine Liver (approximately 40 mg each) were used as a quality control and co-analyzed with the samples during the Se analysis.

A small simulation experiment was undertaken in order to optimize the irradiation and counting conditions for the Mn analysis. The chemical composition of a typical toenail sample was simulated by the addition of representative amounts of Mn (0.002 μg), Na (8 μg), Cl (17 μg), K (5 μg), Ca (32 μg), and Mg (14 μg) in the form of liquid standards to 20 mg sample of ashless paper pulp inside a 0.4 ml high density polyethylene vial.[27] Samples were irradiated for 1 minute or 2 minutes and then counted for 10 to 30 minutes over a two-hour period.

Table 1. One Source Multivitamin cohort

Group	*n*	Age, y	BMI	Smoking status (*n*)		
				Never	Past	Current
Females						
+OS	37	50 ± 13	24.9 ± 4.5	26	10	1
–S	37	50 ± 13	24.8 ± 4.6	25	11	1
Males						
+OS	26	58 ± 12	28.0 ± 4.0	16	9	1
–S	26	58 ± 12	27.9 ± 4.1	16	9	1
Replicates ("Splits")	62					
Reproducibilty QC	20					
Total:	208					

The One Source cohort samples were analyzed for Mn by INAA in August, 2005. The samples used in the earlier Se analysis were re-irradiated in a pneumatic tube system for 90 seconds at a flux of ca. $5.0 \cdot 10^{13}$ $n \cdot cm^{-2} \cdot s^{-1}$. The samples were then allowed to decay for 300 seconds during which time the samples were transferred from the irradiation vial into a clean 0.4-ml polyethylene vial, re-weighed, and pinned to the bottom of the 0.4-ml vial using expanded polyethylene plugs. The samples were then counted at a source-to-detector distance of ca. 1 cm on a HPGe coaxial detector for 600 seconds in order to observe the signal from the shorter lived elements in this "mid" irradiation. Elements observed in this initial count include Cu, Mg, Ti, V, Sc and Al. The detector had a measured relative efficiency of 20% and a FWHM resolution of 1.85 keV at 1.33 MeV. Following this initial count, the transferred sample was allowed to decay for one hour from the end of irradiation and then counted for twenty minutes on the face of one of two HPGe detectors for the Mn analysis. The two detectors had measured relative efficiencies of 27% and 31% and a FWHM resolution of 1.7 keV at 1.33 MeV. The peak areas were determined interactively with Genie ESP spectroscopy software package (Canberra). Transfer of the sample from the irradiation vial was necessary because the Mn signal observed in blank irradiation vials was, on average, ten times greater than the lowest Mn signal observed in the nail samples. Comparator standards were prepared by pipetting approximately 0.25 μg of Mn from a certified Special Mix Standard (High Purity Standards) onto filter paper in 0.4 ml polyethylene vials. Eleven samples of NIST SRM 1577 Bovine Liver (ca. 40 mg each) were used as a quality control and co-analyzed with the samples during the Mn analysis.

Results

The simulated toenail experiment showed that the maximum signal to noise ratio for the ^{56}Mn 846 keV gamma-ray was achieved with a 1 minute irradiation and a 60 minute decay time. The actual irradiation time used for the Mn measurement was 90 seconds in order to increase sensitivity for other elements measured with the same irradiation. In addition to achieving a high signal to noise ratio, the 60 minute decay reduced or eliminated the interference from the 843 keV gamma emitted by ^{27}Mg ($T_{1/2}$ = 9.46 m).

The Se and Mn concentrations of the nail samples were determined using standard comparator INAA. The Se analysis was performed utilizing the ^{76}Se(n,γ)^{77m}Se reaction and measuring the 161.9 keV gamma-ray ($T_{1/2}$ = 17.4 s). The average (n = 12) response function for the 0.5 μg Se comparator standards was $(1.16\pm0.01)\cdot10^5$ counts/μg Se. The average (n = 10) observed Se value in the NIST SRM 1577 Bovine Liver samples was 1.06±0.03 μg/g; the certified Se value in this SRM is 1.1±0.1 μg/g. As in previous studies, a mild correlation was observed between the measured Se concentration and the nail sample mass. The correlation was corrected as described in GARLAND et al.[28] The Mn analysis was performed using the ^{55}Mn(n,γ)^{56}Mn reaction and measuring the 846.8 keV gamma-ray ($T_{1/2}$ = 2.58 h). Two detectors were used to increase sample throughput during the experiment. The average response function for the 0.25 μg Mn comparator standards on detector one (n = 6) was $(1.14\pm0.02)\cdot10^6$ counts/μg Mn and on detector two (n = 10) was $(1.28\pm0.02)\cdot10^6$ counts/μg Mn. The average observed Mn value in the NIST SRM 1577 Bovine Liver samples on detector one (n = 3) was 10.7±0.1 μg/g and on detector two (n = 5) was 10.6±0.6 μg/g; the certified value for Mn in this SRM is 10.3±1.0 μg/g.

The average 95% confidence level minimal detectable amount (MDA) of Se and Mn in the nail samples was 0.8 ng and 0.4 ng, respectively. These values correspond to a limit of detection of 17 ng/g for Se and 8 ng/g for Mn when using the median sample mass of 48 mg. For the 62 individuals for whom a blinded split sample was available, the split sample results were averaged. The average relative standard deviation between the two analyses for these 62 individuals was 4% for Se and 24% for Mn. This difference in variance was also observed in the 20 control nail samples collected from a single individual over the course of a year, the mean Se concentration was 0.87±0.04 μg/g while the mean Mn concentration was 0.11±0.06 μg/g.

The concentration of Se in the nail samples ranged from 0.7 to 3.0 μg/g and the concentration of Mn in the nail ranged from 0.02 to 1.6 μg/g. The mean Se concentration values obtained for the female (n = 35) and male (n = 26) supplementers (+OS) were 1.15±0.13 μg/g and 1.17±0.19 μg/g, respectively, and the mean Se concentrations obtained for the female (n = 37) and male (n = 25) non-supplementers (–S) were 1.01±0.13 μg/g and 0.99±0.17 μg/g, respectively. Females and males in the +OS group were found to have mean toenail Mn concentrations of 0.12±0.14 μg/g and 0.081±0.040 μg/g, respectively, while females and males in the –S group had mean toenail Mn concentrations of 0.16±0.24 μg/g and 0.20±0.34 μg/g, respectively.

In order to account for the non-normal distribution of the nail concentrations, the statistical analyses were performed on the natural log transformed data. The boxplots for the natural log transformed toenail Se and Mn concentration in the +OS and –S groups are shown in Fig. 1. The statistical analysis of the log transformed Se and Mn results was performed by matching each treatment sample (+OS) with its matched control (–S).

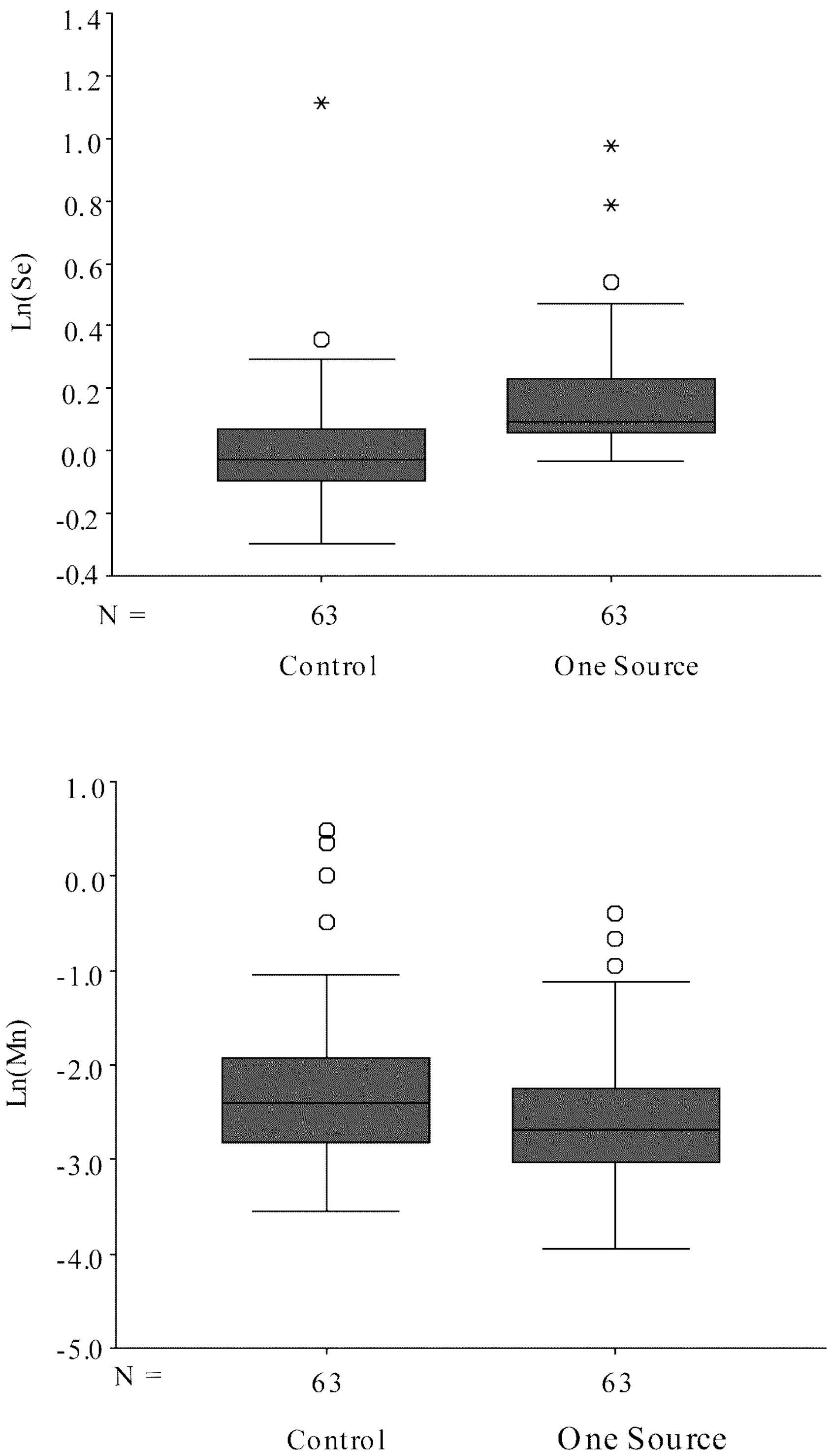

Fig. 1. Boxplots showing the One Source and Control group distributions of log-transformed toenail Se and Mn concentrations

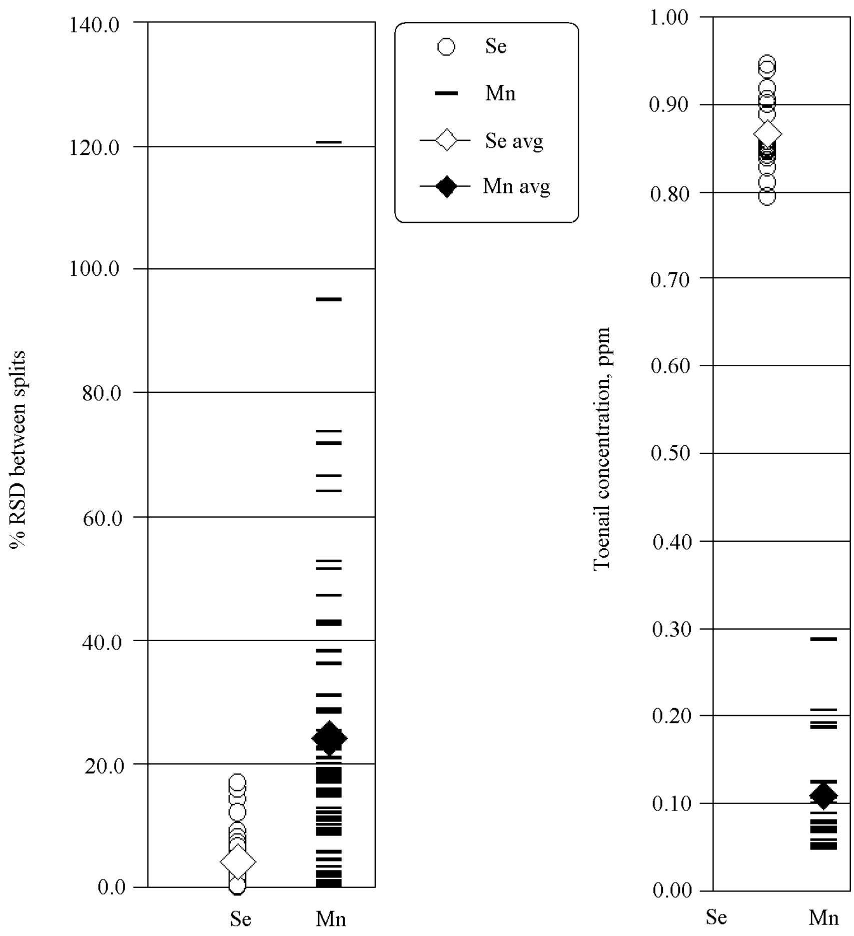

Fig. 2. Variability of Mn and Se results shown in distribution graphs, on left, %RSD's between replicate ("split") pairs, and on right, the concentrations measured in 20 control samples from one individual

This procedure assumes that there is less variability between matched pairs than between unmatched pairs. A mixed statistical model was employed which accounts for random effects, such as element concentration, and fixed variables such as +OS or –S status. Because duplicate measures of one individual are correlated, the correlation must be accounted for in the statistical analysis. A compound symmetry model was used in the Mixed procedure in SAS (SAS Institute Inc., Cary, NC) to account for the 62 duplicate samples. The Mixed model analysis shows, for a two-sided alternative hypothesis, a highly significant difference between the means of the ln(Se concentration) between the +OS and –S group; +OS mean (0.16±0.18) is higher than the –S mean (–0.0114±0.14) with a p value <0.0001. The same analysis indicates a borderline significant result between the means of the ln(Mn concentration) between the +OS and control group ($p = 0.045$). The marginal difference is, however, in the unanticipated direction as the control group has a higher mean value (–2.37±0.81) than the supplement group (–2.56±0.70).

Discussion

Measurement of Se in the nail provided an important internal control for this experiment as the One Source cohort is a subset of the original CTS. In the original study, Se supplementation was observed to yield a significant increase in toenail Se concentration relative to non-supplementers.[26] Because the same result was obtained in this study, we conclude that any misclassification of +OS or –S status by the participants was minimal.

In contrast to Se, the nail did not show a significant, positive response to Mn supplementation. A major difference between the Se and Mn results was, however, the larger variability in the Mn values in multiple samples from a single individual. As noted earlier, the percent relative standard deviations (%RSD) were calculated for the Se and Mn results for each of the 62 pairs of split samples; the mean %RSD for Se was 4% whereas the mean %RSD for Mn was 24%. A similar difference in the variance between Se and Mn was also observed in the 20 control nail samples taken from a single individual over the course of a year; 4% RSD for Se and a 58% RSD for Mn (Fig. 2). The large variance in the Mn concentration in the sample splits and the control nail samples suggests that Mn is not distributed homogenously throughout the sample.

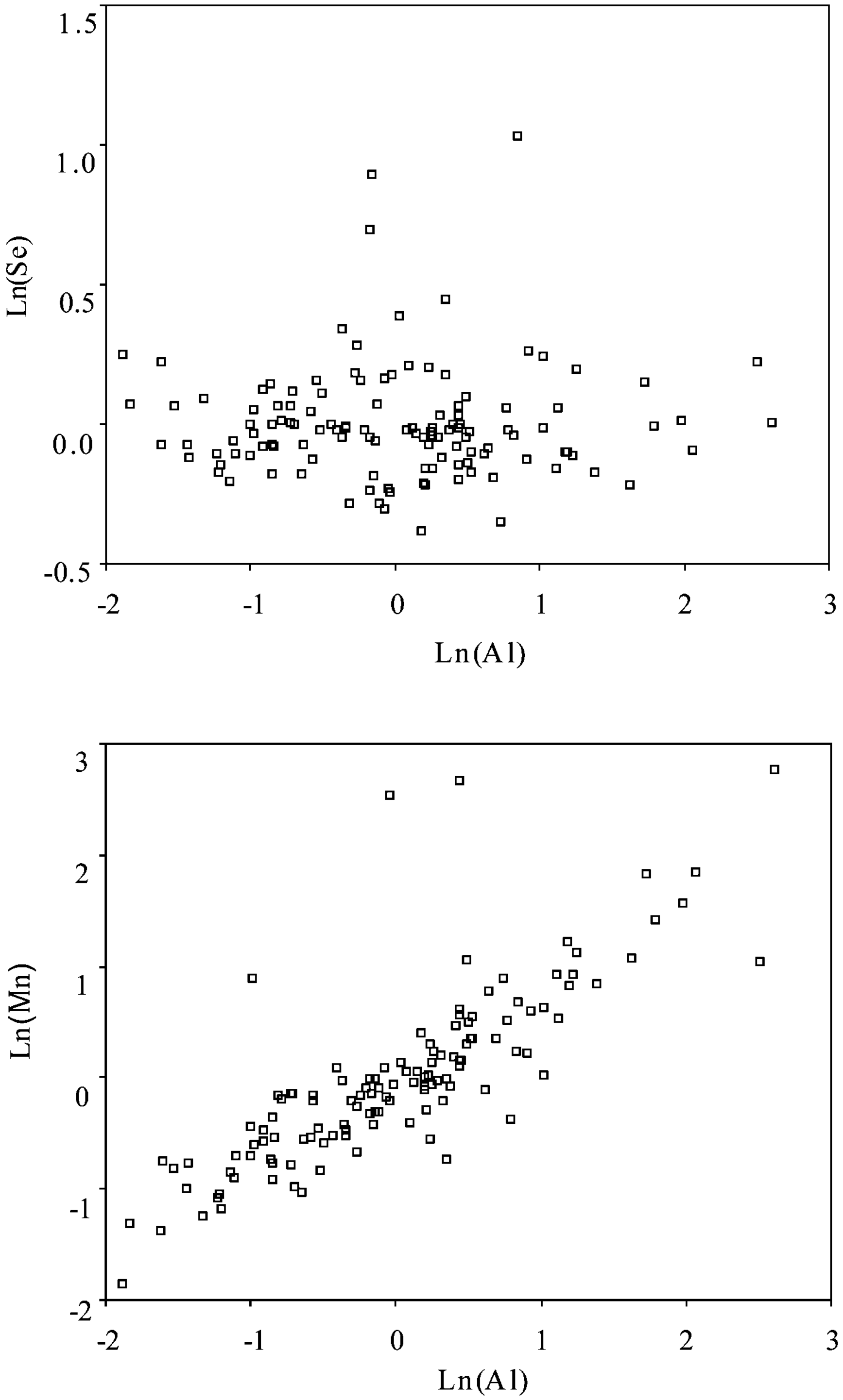

Fig. 3. Scatter plots for Se vs. Al (no correlation, Pearson coefficient –0.032) and Mn vs. Al (positive correlation, Pearson coefficient 0.811)

This observation led us to hypothesize that a portion of the Mn in the nail originates from an external source. That is, while the nail samples were cleaned and visually inspected in an attempt to remove exogenous material, the procedure is not removing all contamination. To test this idea, the relationship between the Al signal (counts/μg) observed in the short count of the "mid" irradiation and the nail Mn and Se concentration was examined. Aluminum is widely distributed in the terrasphere and its presence in the nail suggests exogenous contamination. As can be seen in the plots of the log transformed Mn and Se concentrations versus the log transformed Al signal presented in Fig. 3, Mn ($p<0.0001$) is highly correlated with the Al in the nail whereas Se ($p=0.67$) is not. The strong correlation between Mn and Al may indicate that a significant portion of the Mn measured in the nail originates as terrestrial contamination.

The Mn and Se data were reanalyzed using a covariant model that normalized the Al concentration between the +OS and –S groups. Again, a marginal difference was observed ($p=0.055$) in the mean ln(Mn concentration) between the two groups with a higher value observed for the –S group.

Conclusions

In agreement with previous work, this study demonstrates that the toenail is an excellent biomonitor for Se intake. In contrast, the nail does not appear to be an appropriate biomonitor for Mn, since no increase in toenail Mn was observed with supranutritional consumption of this trace nutrient. Our hypothesis is that, for practical purposes, any such increase in metabolically deposited Mn is masked by a relatively large amount of persistent exogenous contamination.

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